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Isolation of aggresomes and other large aggregates

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Molecular chaperones and the ubiquitin-proteasome system (UPS) play an important role in handling soluble abnormal polypeptides that arise as a result of misfolding, damage or mutations. However, under certain conditions these systems fail to repair or destroy abnormal species, leading to formation of small cytoplasmic aggregates. Mechanisms of intracellular protein aggregation attract growing attention because of their relevance to a number of neuropathological conditions. In many major neurodegenerative diseases, such as Amyotrophic Lateral Sclerosis, Alzheimer's Disease, Parkinson's Disease and Huntington's Disease, the pathology and the eventual death of specific neuronal populations occur due to accumulation of certain abnormal polypeptides, which can form insoluble aggregates (for review see (Sherman and Goldberg 2001)).

Recently, it was discovered that special machinery has evolved that transports small protein aggregates in a microtubules-dependent manner to the centrosome, forming an organelle called aggresome (Chung, et al. 2001, Corboy, et al. 2005). The aggresome serves as a storage compartment for protein aggregates, and could be actively involved in their refolding and degradation. In fact, major chaperones, like Hsp70 or Hsp27, and components of the UPS are recruited to the aggresome (Ahn and Jeon 2006, Garcia-Mata, et al. 1999, Kovacs, et al. 2006, McNaught, et al. 2002). Furthermore, recently it was demonstrated that autophagic clearance of protein aggregates also occurs in association with the aggresome (Garcia-Mata, et al. 2002, Olzmann and Chin 2008, Pankiv, et al. 2007).

There is a notion in the field that aggresome formation represents a protective cellular response to a buildup of aggregating abnormal polypeptides under the conditions when chaperones and UPS machineries fail to handle abnormal species (Olzmann and Chin 2008, Tanaka, et al. 2004). Indeed, it was reported that there is a close correlation between aggresome formation and cell survival (Taylor, et al. 2003). Furthermore, toxicity of abnormal proteins is strongly enhanced by inhibition of the microtubule-dependent transport, which is required for aggresome formation. In line with this concept, inhibition of aggresome formation was recently suggested as an approach to enhance the cytotoxicity of proteasome inhibitors to facilitate their anti-cancer activity (Nawrocki, et al. 2006, Piazza, et al. 2007).

Beside aggresome formation other protein aggregation pathways also appear to exist in mammalian cells. For example, mutant glial fibrillary acidic protein (GFAP) expressed in cells seems to be unable to form aggresomes, and usually forms small multiple aggregates all around the cytoplasm (Quinlan, et al. 2007). In another example, we have recently demonstrated that while synphilin 1, a protein associated with Parkinson's disease, forms aggresome, its mutant form lacking an ankyrin repeat domain can only form multiple cytoplasmic aggregates (Zaarur, et al. 2008). To understand the aggresome response it is critical to use clear mechanistic criteria of aggresomes. The characteristic features of

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aggresome that discriminate them from other types of protein aggregates include the microtubules-dependence of aggresome formation and its co-localization with the centrosome.

A number of factors have been implicated in aggresome formation. For example, a microtubule-associated histone deacetylase HDAC6 was shown to interact with cytoplasmic aggregates of ubiquitinated proteins via its ubiquitin-binding BUZ domain, and facilitate their association with the dynein motor protein that drives this cargo to the aggresome (Kawaguchi, et al. 2003). Other proteins also play a role in aggresome formation, e.g., PLIC, ataxin 3 (Burnett and Pittman 2005, Heir, et al. 2006), p62/sequestosome (Donaldson, et al. 2003, Lim, et al. 2005, Seibenhener, et al. 2004) and Parkin (Lim, et al. 2006). Nevertheless our current knowledge about the mechanisms of aggresome formation is very limited. Among the major questions in the field are: (i) how the aggresome machinery recognizes protein aggregates and distinguishes them from monomeric abnormal proteins; (ii) how these aggregates are recruited to microtubules; (iii) how small aggregates in the aggresome are kept together; (iv) how aggresome formation suppresses the toxicity of protein aggregates.

One of the approaches to address these questions would be to identify factors associated with the aggresome-forming abnormal polypeptide.

We have developed a novel method for isolation of aggresomes and other types of aggregates, utilizing a recently established yeast model of aggresome formation (Wang 2007). In this model we used as a substrate a fragment of huntingtin, a pathological protein that causes Huntington's disease. Upon expansion of its polyglutamine (polyQ) domain, huntingtin becomes toxic and aggregation-prone. An important mechanistic insight into the aggresome machinery was the finding that aggresome formation by the huntingtin fragment requires a distinct proline-rich region of this protein, which serves as a special aggresome-targeting signal (Wang, et al. 2009). Accordingly, the polyQ polypeptide without the proline-rich region (103Q) formed multiple toxic aggregates, while the polyQ polypeptide with this domain (103QP) formed typical non-toxic aggresome. This model allows separately isolating aggresomes and multiple aggregates and analyzing differences in their composition.

Isolation of aggresomes from yeast

Strains and plasmids

Wild type strain W303 (*MATa ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-52 can1-100 ssd1-d*). The pYES2-based vector for expression of 103QP and 103Q constructs under control of the *Gall* promoter was described previously (Meriin, et al. 2001).

Antibodies—Mouse anti-FLAG monoclonal antibody was purchased from Sigma (St. Louis, MO). AffiniPure Rabbit anti-mouse and goat anti-rabbit IgGs (H+L) were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Yeast growth and induction—Grow yeast cells overnight at 30°C on selective minimal medium with 2% glucose. In the morning wash cells twice in PBS and transfer into the selective media with 2% galactose for six hours.

Isolation of Aggresomes

1. Grow yeast to a logarithmic phase, and collect cells by centrifugation. To 1 gram of wet cells add 1 ml of lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton

X-100, 5 mM PMSF, 1 mM benzamidine, 5 μ g/ml of each: leupeptin, pepstatin A and aprotinin) and disrupt with glass beads (Sigma, G8772) using Bullet Blender (NextAdvance, Inc.). This step and all the following processes are conducted at 4°C.

2. Centrifuge cell lysates at $1000 \times g$ for 2.5 min to remove the debris. Collect the supernatant, adjust total protein concentration in all samples. Load onto the 2.5 cm (diameter) $\times$ 10 cm gel filtration column (50 ml Sephacryl S-400 HR) with a cut-off range of about 8 MDa. Collect 1.7 ml fractions at 0.85 ml/min flow rate. Analyze fractions by a fluorescent microscope for the presence and abundance of the GFP-labeled aggregates. Combine 4 fractions with the highest content of the aggresomes. As a control, use the corresponding fractions from the lysates of cells with vector only.
3. Transfer the pooled fractions into a 15 ml test tube. Add the primary antibody (mouse anti-FLAG IgG) to the pooled fractions to achieve a final concentration of 70 μ g/mL and incubate the solution for 1.5 h with rotation. Of note, if aggresomes of a distinct protein are isolated, corresponding primary antibodies should be used for decoration of aggregates. Add the secondary antibody (rabbit anti-mouse IgG) to a final concentration of 140 μ g/mL and incubate the solution for 1h. Add the tertiary antibody (goat anti-rabbit IgG) at 300 μ g/mL and incubate the solution for 1.5h.
4. Overlay samples on the 2 ml of the 50% sucrose dissolved in the same lysis buffer in 15 ml test tubes. Spin for 2.5 min at $600 \times g$. Aspirate the supernatant and store the purified aggregate pellet at -20°C .

COMMENTARY

Background Information

Multiple attempts have been undertaken to identify proteins associated with various types of protein aggregates, including aggresomes; most have employed two-hybrid screens (Faber, et al. 1998, Kaltenbach, et al. 2007). The two-hybrid approach, however, does not allow a comprehensive search for proteins sequestered in various types of aggregates, since some of these proteins may interact only with the soluble forms of abnormal polypeptides, or be recruited via indirect interactions.

Another approach would be to isolate aggresome and determine its composition, since factors involved in various stages of aggresome formation, as well as factors involved in the aggresome-mediated cell protection, may physically associate with aggresome. Broad analysis of the components associated with aggresome and other types of aggregates is hampered by the difficulties in isolation of aggregates. These difficulties, which include extreme heterogeneity of size and charge of the aggregates, preclude application of conventional biochemical methods to the purification of aggregates, e.g., gel filtration or ion-exchange chromatography. In addition, enormous sizes of aggregates practically preclude using various types of affinity chromatography for their isolation. In fact, aggregates are washed-out from affinity chromatography columns due to a shear force upon wash, even if they are covalently cross-linked to the beads. Previously, isolation of aggregates was performed by utilizing the ionic detergent insolubility of amyloids (Doi, et al. 2004) or density gradient fractionation (Suhr, et al. 2001). While these methods may be useful to address certain questions about the structure of protein aggregates, they are inadequate for identification of the aggregate-associated proteins. In fact, SDS treatment causes dissociation of most of associated proteins, while density gradient isolation yields a high fraction of non-specifically associated polypeptides. Another method using a fluorescence-activated cell sorter to separate GFP-tagged aggregates overcame some of

these problems. However, the cell sorter approach has several obvious limitations: it requires very large and highly homogeneous aggregates, and provides low yields (Mitsui, et al. 2006). Furthermore, this method is not applicable to the isolation of aggregates from clinical samples.

Our method of aggresome isolation is based on affinity purification without involvement of a solid phase. It allows isolation of aggresomes under mild conditions that preserve associated proteins. The yields are sufficient for a broad proteomics study.

Following yeast cell lysis, the unbroken cells and cell debris are sedimented by centrifugation, which leaves aggresomes and large aggregates in the supernatant. The first step is separation of the aggresomes from soluble mono- and oligomeric forms of the same polypeptide as well as from soluble cellular proteins using gel filtration and collecting void volume fractions highly enriched with aggresomes. The cut-off range of the column is chosen to yield in void volume only very large structures, including aggresomes and other types of large aggregates.

The second and the main isolation step employs an approach eliminating the need for a solid support in affinity purification (Fig. 1). We first decorated aggresomes with a primary antibody (anti-FLAG, since our aggresome-forming polypeptides were FLAG-tagged). Then, we built a 3-D antibody mesh that incorporated aggresomes by consequent additions of an excess of secondary antibody, and finally of an excess of tertiary antibody. As a result of the consecutive incubations, the aggregates became impregnated into an IgG mesh (Fig. 2). The size of this mesh is much higher than the sizes of free large intracellular particles, like components of cytoskeleton that remain in the supernatant after the first centrifugation. Therefore, the mesh could be effectively separated from other large particles by a very low speed centrifugation through a dense sucrose cushion. In fact, since the centrifugation through the cushion was run at a speed lower than the speed used to clarify yeast lysates before gel filtration, even the largest particles un-associated with the IgG mesh did not sediment under these conditions, and remained in supernatant.

The method allows isolation of aggresomes and other types of protein aggregates together with associated proteins. Indeed, we identified more than 30 associated proteins, including a set of molecular chaperones, several glycolytic enzymes, components of the ubiquitin-proteasome machinery, and other polypeptides (Wang 2007). Beside aggresome, this approach can be applied to isolation of any types of large structures, like other types of aggregates, components of cytoskeleton, and even organelles.

Critical parameters

Upon adaptation of this methodology to isolation of various types of structures one should consider that it involves two centrifugation steps. At the first centrifugation extremely large complexes and unbroken cells are removed, and structures of interest should remain in supernatant. Then these structures are incorporated into the IgG mesh, become much larger, and could sediment at lower speeds through the dense sucrose cushion. The critical consideration is that in order to avoid contamination with unrelated complexes, the speed of the first centrifugation must be higher than the speed of second centrifugation. This speed difference ensures that the unrelated complexes remain in supernatant while the IgG-impregnated structures sediment.

To separate the aggresome and the associated proteins from the IgG molecules, biotinylated IgG could be used in the experiment allowing their efficient removal after solubilization of pellets using streptavidin beads.

Anticipated Results

The described method provides yield of about 2mg of aggresome preparation from 1 g of yeast protein. The first step of size exclusion chromatography purifies aggresomes about 7-fold, and the second step of immunoisolation purifies them about 20 fold.

Time Considerations

Yeast growth and collection of cells takes 24 hours. The entire purification procedure should take one working day. At the end of the day, purified aggresome fractions could be frozen and stored. Analysis of the isolated fractions by 2-D SDS-PAGE should take another day. One extra day could be spent for the removal of the antibodies using streptavidin column.

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Fig. 1.
Scheme of steps 2 and 3 of aggresome isolation.

Fig. 2.
Appearance of aggregates before (A) and after (B) formation of the IgG mesh.